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2. Patent application number — 25 FEB 2002 — **0204348.7**
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Patents ADP number *(if you know it)*

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If the applicant is a corporate body, give the country/state of its incorporation — GB

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4. Title of the invention — Novel Complexes for Inducing an Immune Response

5. Name of your agent *(if you have one)* — MEWBURN ELLIS

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Patents ADP number *(if you know it)* — 109006

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Signature: Mewburn Ellis

Date: 25 February 2002

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DUPLICATE

Novel Complexes for Inducing an Immune Response

The present invention relates to novel complexes for inducing an immune response in an individual. Particularly, but not exclusively, the invention relates methods of inducing an immune response against one or more antigens using Fas ligand as an adjuvant.

Immune surveillance by both the innate and acquired arms of the immune system plays a part in eliminating some transformed cells. However the protection afforded is less than previously thought [1]. The frequent appearance of tumours in humans illustrates the fact that many tumour cells are ignored, develop mechanisms to escape, or at least tip the balance of an immune response preventing their elimination. Fas Ligand (FasL), a member of the TNF family of proteins, is well characterised for its role in triggering apoptosis. Expression of Fas ligand by activated lymphocytes allows them to kill target cells expressing Fas [2]. However, expression of FasL by these cells is a two edged sword as activated lymphocytes themselves express Fas and become susceptible to death. The Fas/FasL axis thus limits

an immune response and is a major player in the process of activation induced cell death [3].

FasL expression is normally tightly restricted to activated lymphocytes. However, it has been demonstrated that some non-lymphoid tissues such as the eye and testis can express FasL [4-6]. This has been proposed to underlie the immune privilege enjoyed by these organs, in effect allowing them to kill infiltrating lymphocytes i.e. "kill the killers". Shortly after these initial reports some tumours were demonstrated to express FasL and this was proposed to be a mechanism used to escape an immune response [7,8]. These findings are now the subject of much debate as forced expression of FasL on tumours or transgenic expression in organs such as the pancreas often leads to a brisk neutrophil infiltration and elimination of tumour or damage to the transgenic organ [9].

In this study the inventors show that the molecule Fas ligand, behaves as an adjuvant to generate an anti-tumour response. For the first time, the inventors show that the immunity is mediated by tumour-specific antibodies, which can recognise and lyse the tumour cell line. The

determination that this is an antibody based immunity suggests the potential for a long term tumour immunity. Following this work, the inventors have appreciated that this system when used to generate tumour-specific antibodies may help in the definition of new tumour antigens, and be valuable for the design of better vaccines and therapies of tumours, e.g. melanoma.

Specifically, the inventors have used the B16F10 melanoma model to study the effects of FasL expression. This tumour is poorly immunogenic and thus a good model to test strategies to enhance tumour immunity. They found that mice reject tumour cells transfected with FasL and go on to develop tumour specific immunity. Unlike previous studies where immunity was shown to be mediated by CD8 lymphocytes, this immunity can be transferred by serum. Here the inventors show that immune responses directed to melanocyte differentiation antigens, are indeed induced by FasL expressing tumour cells.

At its most general, the present invention provides materials and methods for inducing an antibody immune response in an individual against an antigen, e.g. a tumour

associated antigen, using FasL as an adjuvant. Further, the present invention provides screening methods for identifying specific tumour associated antigens and methods for producing specific monoclonal antibodies for use in the treatment of cancer.

Thus, in a first aspect, there is provided an immunocomplex comprising a tumour associated antigen and FasL. The tumour associated antigen and FasL may be in the form of nucleic acid, which may be translated in an individual to produce a fusion protein comprising both the tumour associated antigen and the FasL polypeptides. Alternatively, the immunocomplex may comprise the tumour associated antigen and the FasL as a fusion protein. In a preferred embodiment, the tumour associated antigen is provided by transfecting a tumour cell with FasL such that the ligand is expressed by the transfected cell along with the tumour associated antigens. The inventors have illustrated this aspect of the invention by providing a melanoma cell transfected with FasL.

The invention further provides a pharmaceutical composition comprising said immunocomplex according to the first aspect of the invention.

In a second aspect of the present invention, there is provided a method of inducing an immune response in an individual against a tumour cell, said method comprising the steps of

(a) obtaining a tumour cell from said patient;

(b) transfecting said cell with FasL such that said cell expresses FasL; and

(c) administering said transfected tumour cell to said individual so as to induce an antibody immune response against said tumour.

In a third aspect of the invention, there is provided a screening method for identifying tumour-specific antibodies, said method comprising the steps of

(a) transfecting a tumour cell with FasL;

(b) vaccinating a test animal with said transfected tumour cell;

(c) collecting serum from said test animal;

(d) identifying antibodies specific for said tumour cell from the serum.

The method may further comprise the step of producing a pharmaceutical composition comprising said identified antibody. The antibody may be polyclonal or monoclonal. Preferably, the method will further comprise the step of producing monoclonal antibodies using the identified antibody in standard methods.

In a preferred embodiment of the present invention, there is provided a method for identifying specific tumour associated antigens using antibodies raised against an immunocomplex according to the first aspect or the antibodies isolated by a method according the third aspect. The method may comprise the steps of contacting said antibody with a plurality of potential tumour associated antigens obtained from the tumour cell used to vaccinate the test animal. The potential tumour associated antigens may conveniently be displayed using an expression library (e.g. a phage expression library) or on a solid support e.g. a 2d SDS PAGE followed by a western blot. The antibody may then be contacted with the potential antigens and the specific binding between the antibody and the antigen identified by labelling or other routine techniques. The antigen may then be characterised using standard methodology known to the skilled person.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in the text are incorporated herein be reference.

Figure 1

The tumour-protective effect of FasL is dependent on the Fas binding domain and the neutrophil chemoattractant Mip1α

Mice were given 5 x 10^5 live B16F10 cells s.c. either untransfected (*a*) or stably transfected with full length FasL (*b*), full length FasL with a mutation in the Fas binding site ($FasL^{mut}$ in *c*), truncated FasL with no binding to Fas ($FasL^{mut/trunc}$ in *d*) or transfected with truncated FasL ($FasL^{trunc}$ in *e*). Mip1α deficient mice were used for injection in *f*. Tumour growth was measured twice a week over a period of 5 weeks. Numbers in brackets are mice that remained tumour free/ total number of mice.

Figure 2

The serum from protected mice transfers tumour immunity

200μl of serum from protected mice was transferred into naïve C57B/L6 and mice were challenged the following day with 5 x 10^5 B16WT.

Figure 3

The serum from B16FasL immunised mice stains and lyses B16F10 by complementa,*b,c,e,f,gh,i*

Tumour cell lines were stained with serum from protected mice (line) and normal mouse serum (filled area). Second layer antibody in panels *a,f,g,h,i* was anti-mouse Ig, in *b* anti-IgM and in *c* anti-IgG. *d* Serum from protected mice (PMS) was used in a complement lysis assay. Normal mouse serum (NMS) and wells with no complement were included as controls. The experiment is representative of two experiments. ***P*<0.01 student's T test.

Figure 4

Protected mice are able to clear infections with vaccinia expressing gp100 or Trp1

After vaccination + challenge (B) or naïve (A) mice were injected with vaccinia expressing the melanocyte differentiation antigens gp100, Trp-1, Trp-2, Mart-1 or an

irrelevant antigen G2. Five days later viral titers were determined in ovaries. This experiment was repeated twice with similar results. The dashed line represents the limit of sensitivity of the assay.

Figure 5

Dendritic cells mature in the presence of crosslinked FasL and B16FasL

After culture in GM-CSF and Il-4 bone marrow dendritic cells from C57BL/6 mice were matured overnight with crosslinked FasL or irradiated B16WT or B16FasL. BALB/c spleen cells were added to increasing numbers of dendritic cells and proliferation was measured as incorporation of ^{3}H Tritium. Background of proliferating dendritic cells or B16F10 cells alone was substracted. This experiment was repeated three times with similar results. * $P<0.05$ Student's T test.

Fas binding and not the cytoplasmic chain of Fas mediate tumour rejection of FasL expressing tumours.

Counter to initial observations a number of investigators have now demonstrated that FasL transfected tumour cells are more efficiently rejected than FasL negative cell lines upon their injection in vivo. The brisk tumour rejection seems to

be mediated in the main by infiltrating neutrophils. Although it seems clear that once present, neutrophils can kill the tumour cells, little is known about how the neutrophils are attracted in the first place. In a first series of experiments the inventors established a model of FasL mediated tumour rejection using the well-characterised murine melanoma cell line B16F10. Stable cell lines expressing a FasL were established (B16FasL) and injected subcutaneously (sc) into C57BL/6 mice.

Examination of the site of injection revealed a small but clearly palpable swelling at the injected site up to 2 weeks after injection. All 10 mice injected with the parental or "wild type" B16F10 (B16WT) developed tumours (Fig. 1a), whereas 6 of 9 rejected the FasL expressing tumour (Fig. 1*b*) confirming previous studies using the B16 model and other murine tumour models [8]. The inventors next confirmed that this was due to a direct interaction between Fas and FasL by expressing a mutant form of FasL ($FasL^{mut}$), which contains a single point mutation in the extracellular domain, that prevents Fas binding (Y218R) [10]. This mutation abolished rejection and tumours developed in all 10 mice (Fig. 1*c*). This is consistent with previous observations that FasL can

activate neutrophils via Fas and that tumour rejection is prevented in lpr mice that express a mutated non-signalling Fas [11,12]. FasL has a long and conserved cytoplasmic domain rich in proline residues [13,14], which could potentially allow "reverse signalling" via FasL to the tumour cell, perhaps causing the secretion of neutrophil chemoattractants. However deletion of this domain ($FasL^{trunc}$), either alone or in combination with the extracellular mutation had no influence ($FasL^{mut/trunc}$, Fig. 1*d* and *e*), implying that rejection is solely due to Fas-FasL interaction.

Further support for role of neutrophils was obtained when tumours were injected into Mip1α deficient mice. Mip1α has previously been shown by others to act as a neutrophil chemoattractant [15,16]. In agreement with these observations, the ability to reject B16FasL was impaired in Mip1α deficient mice. Although the kinetics of tumour growth were slightly slower for the B16FasL tumour versus the B16WT control 7/10 mice injected with the B16FasL developed tumours suggesting a possible role for this neutrophil chemoattractant in FasL mediated tumour rejection (Fig. 1*f*).

FasL expressing melanoma induces tumour immunity

Of 123 mice vaccinated with B16FasL 56% remained tumour-free (Table 1). When tumour-free vaccinated mice were challenged with 5 x 10^5 B16WT about half of the 69 mice (54%) rejected the second tumour. These results were confirmed using two independent sublines of B16WT . For subsequent experiments it was established that 10 x 10^6 irradiated B16FasL could protect against a second tumour. This has the advantage of preventing the growth of the FasL transfected tumours occurring in 44% of mice given live B16FasL. All of the mice given irradiated B16FasL remained tumour free. 64% of these mice treated with irradiated B16FasL developed tumour immunity (n=50) and rejected tumour when challenged with B16WT. As expected, irradiated B16WT did not give rise to tumour immunity (Table 1), confirming results of other studies [17]. Similar results were obtained using several different clones expressing FasL (data not shown).

Tumour immunity can be transferred by serum, but not by $CD4^+$ or $CD8^+$ T cells.

Mice were vaccinated with B16 FasL and challenged with B16WT tumour. Animals that rejected the tumour challenge were then

selected for further study and are subsequently referred to here as "protected mice". To determine which cells are critical for tumour immunity, protected mice were depleted of $CD4^+$ or $CD8^+$ T cells by administration of depleting antibodies in vivo. Mice were then re-challenged with B16WT. Both $CD4^+$ and $CD8^+$ depleted mice were still able to reject the tumour (Table 2). Similarly the transfer of purified $CD8^+$ or $CD8^+/CD4^+$ lymphocytes from protected mice did not prevent tumour growth. The inventors also tested fresh ex vivo killing of B16WT or targets infected with vaccinia viruses encoding melanocyte differentiation antigens. At a variety of effector target ratios they detected no lytic activity from splenocytes or purified $CD8^+$ lymphocytes (data not shown).

The lack of effect using depleting antibodies was surprising, as it has previously been shown that FasL can prime CD8 T cell responses [18,19]. This prompted the inventors to test for the development of a humoral anti-tumour response. Serum was collected from protected mice, pooled and then injected into naïve mice, which were subsequently challenged with 5×10^5 B16WT. 14/17 mice given serum were protected from challenge, whereas none out of 17

given normal mouse serum developed tumour (Fig. 2). Serum was still protective when obtained 6 months after vaccination and serum from unvaccinated tumour-bearing mice failed to protect (data not shown).

To show that the tumour protection was mediated by antibody the immunoglobulin fraction of the serum from B16FasL treated or control mice was purified over a Protein L column, known to bind a high proportion of murine IgG and IgM. 1.2mg of purified Ig was injected intravenously followed by challenge with either 2 x 10^5 B16WT. In this experiment all four mice given control Ig developed tumour at day 26 whilst 3/4 of mice given serum from protected mice remained tumour free (Fig. 2).

<u>The serum from immunised mice reacts with melanoma antigens and can trigger complement-mediated lysis of B16F10.</u>

B16WT (melanoma line from C57BL/6 mouse strain), K1735 (melanoma line from the C3H mouse strain), MC57 (methylcholanthrene-induced fibrosarcoma cell line from C57BL/6 mice), 293T cells (human embryonal kidney fibroblast), TK^- cells and thymocytes from C57BL/6 mice were stained by indirect immunofluorescence using serum from

protected, tumour bearing or control mice. FACS analysis showed no staining with either control serum or serum from tumour bearing mice, whereas strong staining of B16WT was seen using the serum from protected mice (Fig. 3a). Antibodies were of both IgM and IgG isotypes (Fig. 3b and c), and serum also stained the other melanoma cell line K1735 and MC57 cells (Fig. 3e and *f*) but not 293T, MDCK or thymocytes from C57BL/6 grown in serum overnight. This result indicates that antigens recognised by the polyclonal serum are not due to fetal calf serum present in the culture medium. In a complement lysis assay only protected serum gave significant lysis of B16WT indicating one possible mode of activity in the in vivo protection against tumour challenge (Fig. 3*d*).

Tumour immune mice react to melanocyte differentiation antigens

In a search for antigen specific responses induced by vaccination with B16FasL the inventors took advantage of a panel of vaccinia viruses expressing the melanocyte differentiation antigens gp100, Trp-1, Trp-2, Melan-A/Mart-1 or an irrelevant antigen [20,21]. When injected into naïve mice these viruses replicate and this can be assayed by

counting plaque-forming units from ovaries infected with vaccinia virus. If the mouse has pre-existing immunity against melanocyte antigens carried by the virus then replication will be inhibited and the ovary plaque count reduced. Vaccinia titres measured in ovaries were consistently high in control mice (Fig. 4*a*). The majority of vaccinated mice infected with vac-gp100 cleared the virus or had reduced viral load (4 out of 6, Fig. 4*b*), one vaccinated mouse cleared vac-Trp-1 and viral titres to Mart-1/MelanA were lowered in 2 mice by a factor of 10^3 as compared to controls (Fig. 4*b*). It is possible that B16FasL will also break tolerance to other tumour associated and tumour specific antigens expressed by B16 and the protection the inventors observe may well be improved by such a polyspecific response. The observation that 20% of mice exhibited depigmentation at the site of tumour inoculation (B16FasL) further supports the findings that immune responses to melanocyte antigens were induced in these mice.

B16FasL can mature dendritic cells.

To gain more insight into how FasL expression might help prime tumour specific responses, the inventors examined whether B16FasL could mature dendritic cells (DC). Bone

marrow derived DC's were cultured in vitro for 4 days, matured in the presence of irradiated B16WT, B16FasL, or soluble FasL-FLAG fusion protein. FasL was included either alone or crosslinked with anti-FLAG mAb, then washed and added to allogeneic splenocytes in a 4 day proliferation assay using tritiated thymidine. Activation of splenocytes was improved significantly when DC's were matured in the presence of B16FasL or soluble crosslinked FasL (Fig. 5). Maturation markers such as MHC class II, CD83 or CD86 were also upregulated on DC's upon culture with FasL (data not shown).

Discussion

The immune system has evolved to recognise and kill foreign pathogens. To avoid this, a number of pathogens have developed strategies to escape or limit the immune response allowing them to set up a more productive infection and in some cases persist in their hosts. Tumours present a more difficult problem as most of the genes they express are normal host genes and the selective pressure exerted on the immune system may be less than by microorganisms; tumours will die with their hosts and often develop after reproductive age. Like microorganisms tumours have developed

a number of strategies to evade responses, which can generally be divided, into those preventing recognition and those which allow escape, or tolerance of a response.

FasL has an interesting pedigree in the field of tumour immunology. Although Fas is expressed on a number of non-lymphoid tissues, such as the liver, FasL expression is tightly regulated, being found predominantly on activated lymphocytes. The role of FasL in the immune system is well studied; it is one of the mechanisms used by lymphocytes to kill targets expressing Fas [2], and it has also been suggested that Fas can deliver an activating signal to T cells [22]. In addition, activated lymphocytes which co-express Fas and FasL, become susceptible to apoptosis [3]. The importance of this for the control of peripheral T cell populations is illustrated by the lymphoproliferation and auto-immunity developed by humans or mice with mutations in Fas or FasL [23].

A few extra-lymphoid tissues including the brain, eye and testis have been shown to express FasL. Expression of FasL at such sites is proposed to limit inflammation and confer immunoprivilege by allowing them to kill infiltrating

lymphocytes expressing Fas [4-6]. These observations incited much interest in several fields, most notably transplantation and tumour immunology. Initial observations demonstrated expression of FasL on some tumours where it was proposed to allow them to escape the immune response. Following these observations a number of investigators tested the effects in tumour cells or transgenic organs overexpressing FasL. Often the results were disappointing (from the tumours standpoint), tumour cells or transgenic tissues faired worse than their non-transfected counterparts [9]. The expression of FasL in a number of instances provoked an intense neutrophil infiltrate [18,24]. Neutrophils express Fas and can be activated by FasL to become cytotoxic [25]. It is believed that this neutrophil activation leads to tumour rejection and blocking with soluble Fas-Fc fusion protein reduced neutrophil activation and killing of FasL transfected tumour cells [25]. The inventors results using B16F10 transfected with a FasL mutant which fails to bind Fas confirm these results.

However it is not entirely clear why FasL transfected tumours induce such a brisk neutrophil infiltrate. Previous

studies have suggested a role for IL1β as FasL transfected fibrosarcoma was not rejected in IL1β deficient mice [26]. In addition soluble FasL can also attract neutrophils, although expression of a soluble form of FasL in tumour cells did not lead to their rejection, or to a neutrophil infiltrate [11,27,28]. In this study the inventors tested rejection in mice deficient in MIP1α. These mice were also less efficient at rejecting the tumour suggesting a possible role for MIP1α as a neutrophil chemoattractant in FasL induced tumour rejection [29]. Consistent with this finding is the recent demonstration that membrane-bound FasL leads to increase of mRNA expression of MIP1α and other proinflammtory mediators [30]. As soluble FasL does not induce neutrophil infiltration the inventors examined the role of the cytoplasmic domain of FasL. This proline rich domain of around 70 amino acids is highly conserved between species and can bind the src kinase fyn in vitro [14]. Previous studies have shown a role for the FasL in the proliferation of CD8 cells and also cell cycle arrest of CD4 cells [31,32]. Thus it has been suggested that FasL may deliver a "reverse signal" via the cytoplasmic domain. In the inventors' experiments deletion of the cytoplasmic domain had no effect

on the kinetics of tumour growth ex vivo (data not shown), and likewise had no effect on tumour rejection in vivo.

Although the initial rejection of FasL expressing tumour has been studied extensively little is known about how this affects long term tumour immunity. In two previous studies protective responses to lymphoma or neuroblastoma were induced by treatment with tumour transfected with FasL [18,19]. In both cases the responses were shown by depletion to be mediated by $CD8^+$ cells. In the inventors' experiments depletion of CD8 cells had no effect. Similarly depletion of $CD4^+$ cells from protected animals did not reduce tumour rejection although transfer of CD4 cells and splenic B cells prevented tumour growth in 2/4 animals whilst slowing growth in the other 2/4 (data not shown). The B16F10 melanoma model has recently been shown to belong to the type of tumours that are weakly immunogenic because they grow as nodules "walled off" from the immune system preventing activation of anti-tumour immunity mediated by $CD8^+$ lymphocytes [33]. Thus the B16F10 model is a particularly difficult tumour model to raise an adaptive immune response, and it might be representative of human tumours [34] that are clinically detectable.

To the inventors surprise serum provided almost complete protection against a secondary tumour challenge. The serum from protected mice can recognise surface determinants on B16F10 and the unrelated melanoma cell line K1735. It is impossible at this stage to say how broad the response is or which antigens on the melanoma are responsible for the protection afforded by serum transfer. However, the inventors have appreciated that their model may be used to screen for specific antibodies and/or specific tumour associated antigens which may be used as tumour vaccines. It is possible to produce monoclonal antibodies from the protected animals to define some of these antigens. However the inventors' screen of melanocyte differentiation antigens expressed in recombinant vaccinia virus has identified some antigens. gp100 and Trp-1 responses were detected in the protected mice. Trp-1 can be expressed at the cell surface and passive transfer of antibodies against Trp-1 have been shown to induce rejection of melanoma in vivo [35,36].

How FasL is able to elicit these responses is at present not entirely clear. It seems likely that the effect is multifactorial. Firstly the recruitment and activation of

neutrophils by FasL is likely a crucial event as it not only allows early innate killing of the tumour, but also leads to the secretion of proinflammatory cytokines and further immune activation [37]. Dendritic cells may be recruited by this milieu and induced to take up tumour cells/antigen. Furthermore FasL expressed on the tumour can help mature the DC's leading to increase expression of costimulatory molecules and consequent priming [38]. The use of other proinflammatory cytokines expressed on tumour cells such as GM-CSF, B7-1, MHC class II or cytokines may well work in a similar fashion by inducing effective DC maturation and cross priming [39]. Recent evidence suggests that the innate anti-tumour response mediated by natural killer cells modulates the development of the adaptive immune response [40]. Similarly it is possible that the innate immune response initiated by FasL induces the humoral anti-tumour response found in this study.

Several monoclonal antibodies such as anti-CD20 and anti-HER2 are already used in the treatment of cancer and a larger number are currently undergoing clinical trials [41]. Antibodies are attractive for the treatment of cancer as

they have many modes of action; blocking or stimulation of a cell surface receptor, cytotoxicity via complement or antibody dependent cell mediated cytotoxicity, and finally if inactive on these counts, conjugated antibody can be used to deliver drugs or toxins to cancer cells. Single monoclonals will have their limitations as they put a huge selective pressure on the tumour to lose antigen expression. In order to produce a truly effective therapeutic tumour vaccine/immunotherapy in humans it will probably be necessary to induce responses against multiple antigens using multiple effector mechanisms CD4, CD8 antibody. The inventors' observations here may help in the definition of new cell surface tumour markers and may provide an additional route to stimulate a broader anti-tumour response.

Experimental Procedures

Tumour cells

B16F10 were obtained from Professor I. Hart (London, UK) and from Prof. R. Zinkernagel (Zürich, Switzerland). Cultures of the melanoma cell line B16F10 were maintained in RPMI (Sigma) supplemented with 10% foetal calf serum (FCS), L-

glutamine, penicillin-streptomycin. The K1735 is a melanoma cell line from C3H strain of mice and was obtained from Prof I. Fidler (University of Texas). MC57 is a methylcholanthrene-induced fibrosarcoma cell line from C57BL/6 strain of mice.

FasL was cloned into the pEGFP-C1 vector (Clontech) placing GFP at the amino terminus of FasL. Deletion mutants lacking the cytoplasmic chain of FasL (lacking 72 amino acids) and/or a single point mutation that abolishes binding to Fas (Y218R) were created by PCR and subsequently cloned into the same vector. B16F10, cells were transfected with the above constructs with DMRIE (Gibco) and selected on 1.5 mg/ml G418, single-cells were sorted by flow cytometry and cloned. Clones were screened for FasL expression using Nok-1 (Pharmingen) by FACS. The in vivo results are representative of several different clones. $5x10^5$ live or $1x10^7$ irradiated tumour cells were injected into C57BL/6 mice and tumour growth measured.

Depletion/ Transfer of $CD4^+$ and $CD8^+$ lymphocytes

Pairs of hybridomas secreting anti-CD4 (YTS 191.1.2, YTS 3 3.1.2, both rat IgG2b) and anti-CD8 (YTS 169.4.2.1, YTS 156.7.7, both rat IgG2b) were used for depletions as

described previously [42]. 100µg of the pair of anti-CD4 or anti-CD8 antibodies injected intraperitoneally 3 and 1 day prior to injection of 5 x 10^5 B16WT. One day after the last injection less than 1% of $CD4^+$ or $CD8^+$ were detected in peripheral blood by FACS.

Transfer of $CD4^+$, $CD8^+$, B cells and serum

$CD4^+$ and $CD8^+$ lymphocytes from spleen and inguinal lymph nodes were purified by positive selection using either directly conjugated beads to anti-CD4, anti-CD8 (Miltenyi Biotec) or using antibodies against CD4 (Pharmingen), CD8 (Caltag) or B220 (Pharmingen) followed by anti-rat-beads. Purity of CD4, CD8 was between 70% for $CD8^+$ and 95% for $CD4^+$ cells. 10 x 10^6 CD4 and 5 x 10^6 CD8 per mouse were re-injected intravenously (i.v.) the day of purification. To obtain serum blood was clotted at 37°C for 1h, then kept at 4°C for at least 1h, spun at 20000g for 10min. 200µl of serum per mouse was reinjected i.v. Purification of the Ig fraction of serum was performed with a protein L column (Pierce) according to the manufacturer's protocol. Protein L binds to the κ light chain and allows purification of IgM and IgG and possibly other murine antibody isotypes [43]. 1.2mg of the Ig fraction was re-injected per mouse. For all

transfer experiments mice were challenged the day following transfer with 5 x 10^5 B16WT.

Proliferation assay with dendritic cells

Murine dendritic cells were isolated from bone marrow. Erythrocytes were depleted with lysis buffer (Flowgen). Cells were cultured with RPMI 5% FCS (Hyclone) with 500U/ml Il-4 (Peprotech) and 1000U/ml GM-CSF (Peprotech). Cultures were fed on day 2 and day 4 by aspirating off medium and adding fresh medium. On day 4 FasL-flag was either added in soluble form (FasL-flag), crosslinked with 5μg/ml of anti-flag or as membrane-bound form on irradiated (12000rad) B16F10 stably transfected with FasL overnight. Allogeneic proliferation was set up on day 5 by culturing 10^5 BALB/c spleen cells with 10^4 C57BL/6 dendritic cells that have been matured previously in the presence or absence of FasL. Four days later proliferating cells were pulsed with ^{3}H-Tritium and radioactive incorporation was measured.

Vaccinia assay

Mice that were previously treated with B16FasL or not were infected intraperitoneally with 2 x 10^6 PFU of vaccinia expressing different melanocyte antigens or control

antigens. Both ovaries were harvested at indicated time points, and the vaccinia titres were determined on MDCK TK^- monolayers as described previously [44].

Flow cytometry and complement lysis assay

FACS staining with serum was performed at a concentration of 1/100, controls serum obtained from untreated or non-immunised mice with tumours. Second layer antibodies included anti-mouse Ig (Dako), anti-mouse IgM (Sigma) or anti-mouse IgG (Sigma). For the complement assay B16F10 cells were labelled for 90min with ^{51}Cr, washed thoroughly and plated out at 2.5×10^4 per well. Pooled serum from protected mice or normal mouse serum was added at 1:5 dilution for 1h, washed off, then rabbit complement (Low-Tox Cedarlane, Canada) was added at 1:10 dilution for 3 hours in a total volume of 80μl. 35μl was harvested and counted on a beta counter.

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Table 1
The primary B16FasL is rejected and induces tumour immunity

Pretreatment/primary challenge (tumour type)	primary[a] challenge	secondary[b] challenge with B16WT
B16WT	6% (*n*=34)	N/A
B16FasL $5*10^6$ live	56% (*n*=123)	54% (*n*=89)
B16WT $10*10^6$ irradiated	N/A	10% (*n*=50)
B16FasL $10*10^6$ irradiated	N/A	64% (*n*=50)

[a]Mice were either injected with 5×10^5 B16FasL or 10×10^6 irradiated B16FasL.
[b]Four to eight weeks later tumour-free mice were challenged with 5×10^5 B16F10 wild type tumour.

Table 2
CD8 lymphocytes do not confer protection in B16FasL immunised mice

		Experiment 1 (tumour free mice)	Experiment 2 (tumour free mice)
a	Isotype control depletion	4/4	5/5
	CD4+ depletion	4/4	6/6
	$CD8^+$ depletion	4/4	6/6
b	Transfer of $CD8^+$	0/3	0/4
	Transfer of $CD4^+$ and $CD8^+$	0/3	ND
	Transfer of $CD4^+$ depleted splenocytes	ND	0/4

a Protected mice were treated twice with a pair of αCD4 or αCD8 depleting antibodies, then challenged the following day with 5×10^5 B16WT. Tumour growth was monitored over a period of 5 months.
b Lymphocytes were transferred from protected mice into naïve recipients, then challenged with 5×10^5 B16WT. Tumour growth was monitored over a period of 8 weeks.

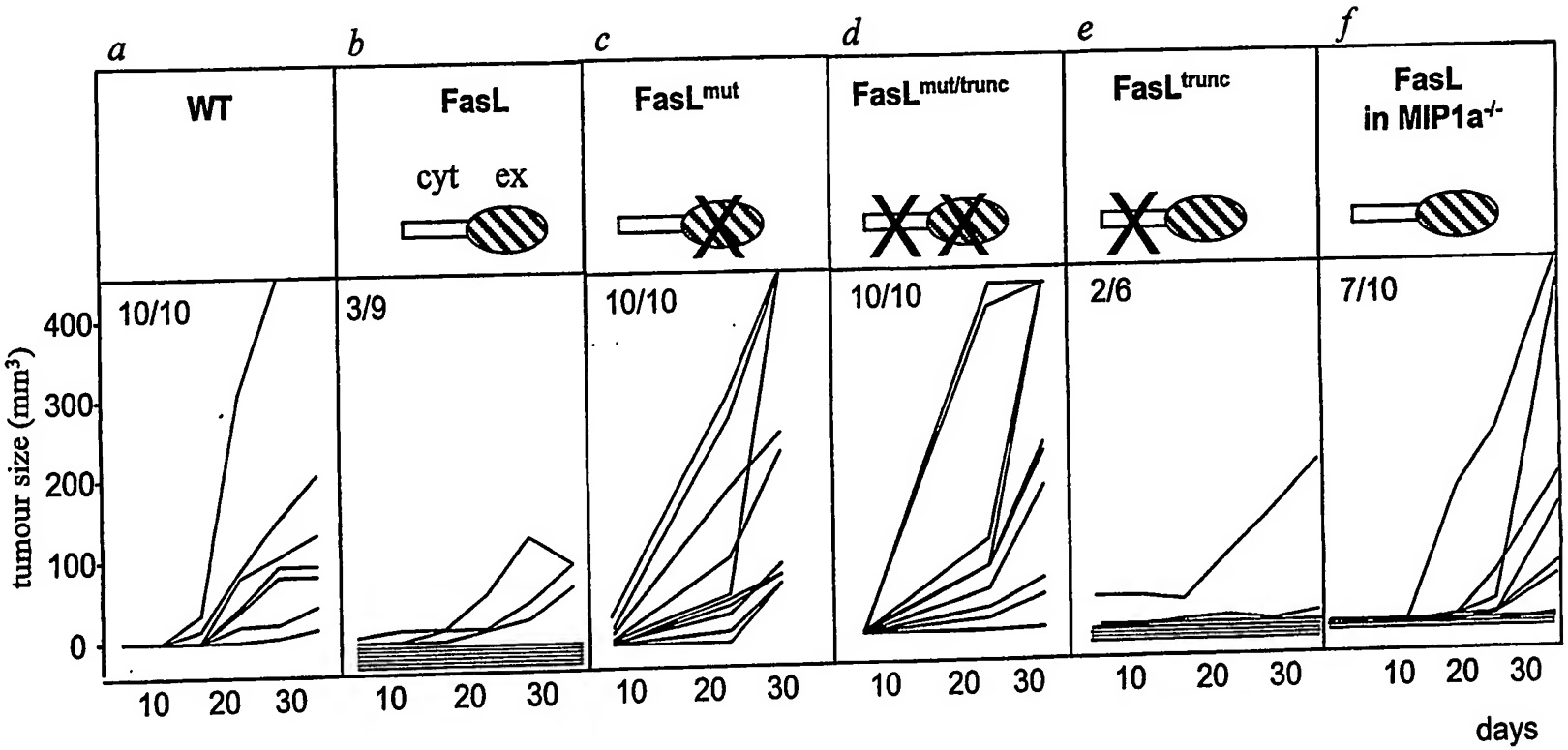

a
b
c
d
e
f
WT
FasL
cyt ex
FasL^mut
FasL^mut/trunc
FasL^trunc
FasL in MIP1a^-/-
10/10
3/9
10/10
10/10
2/6
7/10
tumour size (mm³)
400
300
200
100
0
10 20 30
days

Fig. 1

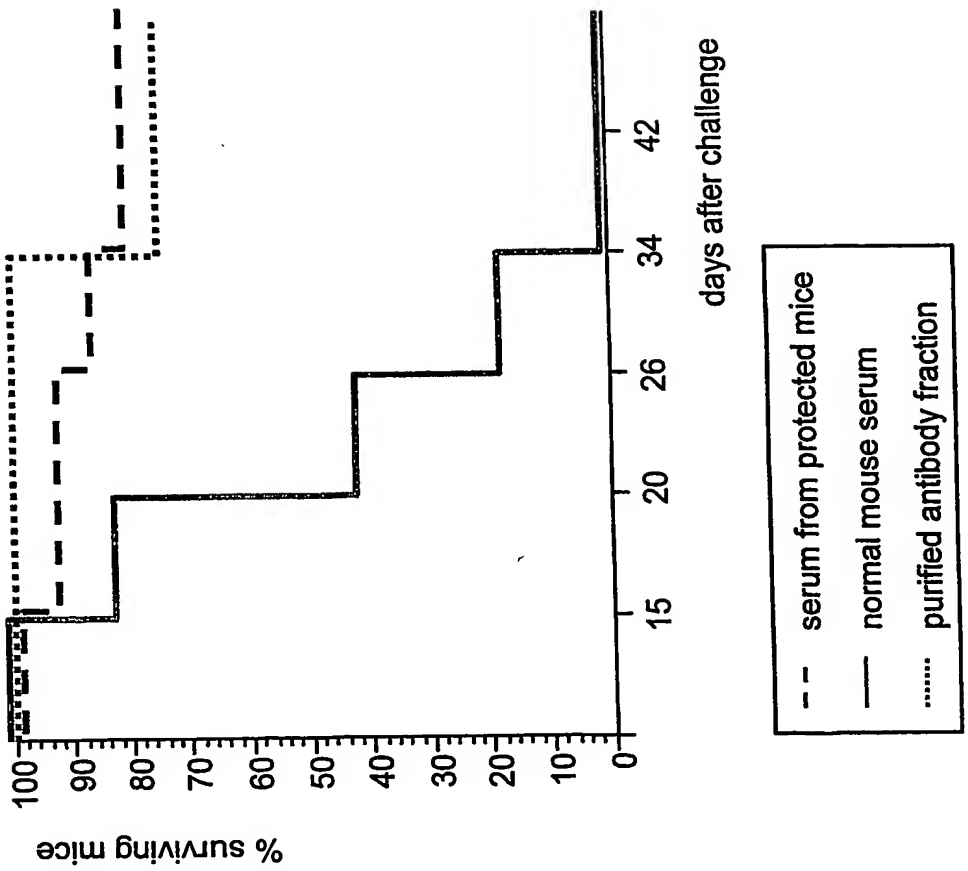

% surviving mice
100
90
80
70
60
50
40
30
20
10
0
15
20
26
34
42
days after challenge
serum from protected mice
normal mouse serum
purified antibody fraction

Fig. 2

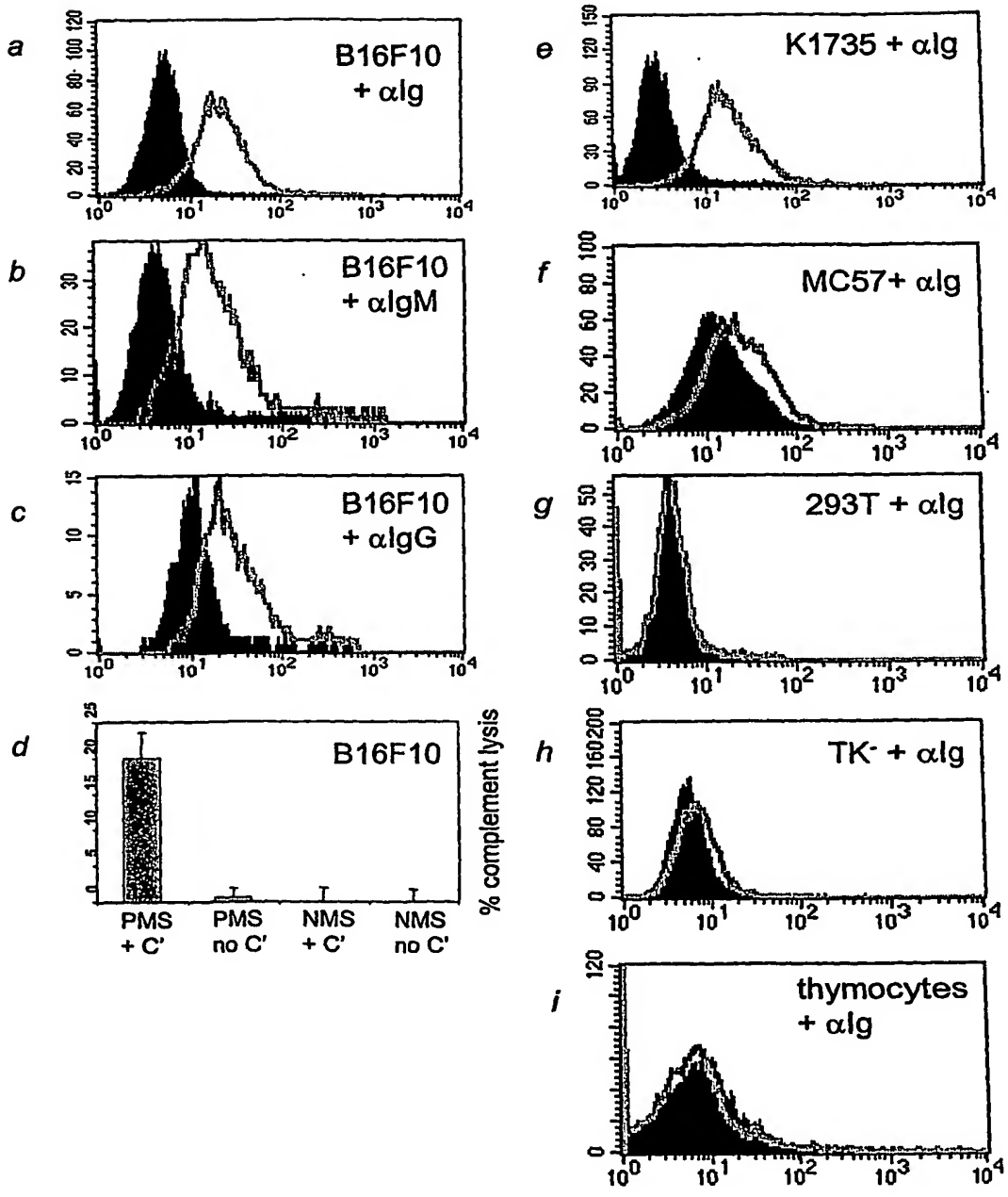

a
B16F10
+ αIg
b
B16F10
+ αIgM
c
B16F10
+ αIgG
d
B16F10
% complement lysis
PMS
+ C'
PMS
no C'
NMS
+ C'
NMS
no C'
e
K1735 + αIg
f
MC57+ αIg
g
293T + αIg
h
TK- + αIg
i
thymocytes
+ αIg

Fig. 3

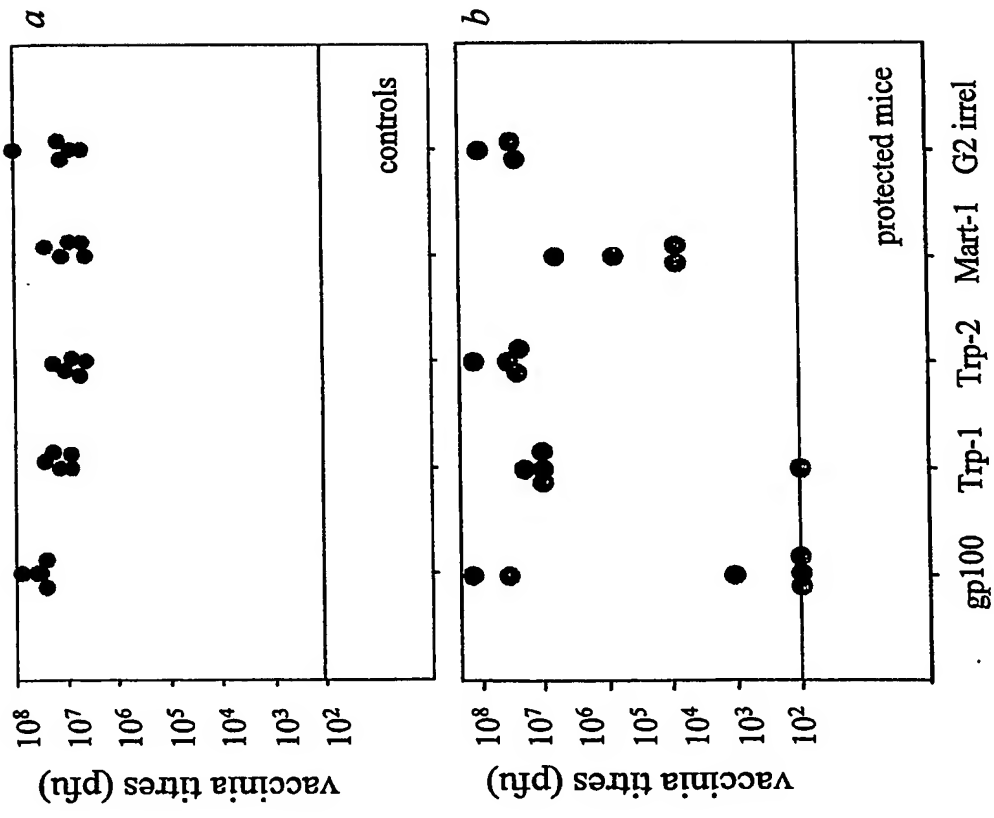
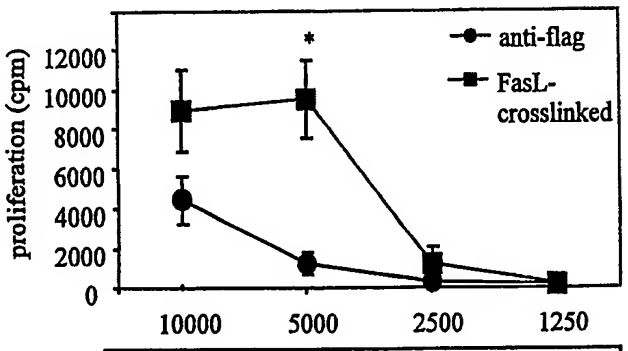

a
b
controls
protected mice
vaccinia titres (pfu)
vaccinia titres (pfu)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
gp100
Trp-1
Trp-2
Mart-1
G2 irrel

Fig. 4

A


proliferation (cpm)
12000
10000
8000
6000
4000
2000
0
10000
5000
2500
1250
anti-flag
FasL-crosslinked
*

B

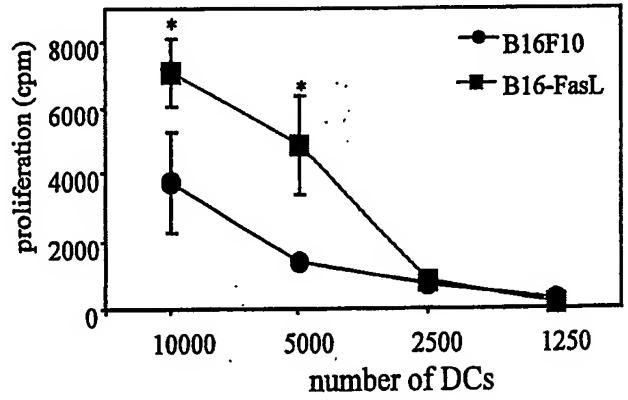

proliferation (cpm)
8000
6000
4000
2000
0
10000
5000
2500
1250
number of DCs
B16F10
B16-FasL
*
*

Fig. 5